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Denaturation and covalent network formation of wheat gluten, globular proteins and mixtures thereof in aqueous ethanol and water

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Abstract

Food processing often includes heating and/or exposure to solvents as unit operations. Here, the impact of heating at 100 °C in water or aqueous ethanol [10 or 50% (v/v)] on denaturation and covalent network formation of three model proteins [bovine serum albumin (BSA), soy glycinin and wheat gliadin] was examined. Already at room temperature 50% (v/v) ethanol induced disulfide cross-linking between BSA proteins. Increased ethanol concentrations reduced heat-induced polymerization of soy glycinin and wheat gliadin. The use of aqueous ethanol limited the extent of β-elimination, sulfhydryl-disulfide exchange reactions and sulfhydryl oxidation. Gliadin and soy glycinin had higher colloidal stability in 50% (v/v) ethanol than in water. The conformation of BSA and soy glycinin already changed at lower temperatures in 50% (v/v) ethanol than in water. In all media, different proteins influenced each other's denaturation and/or polymerization. During heating in water but not in 50% (v/v) ethanol, gliadin-BSA and gliadin-soy glycinin mixtures polymerized more than expected than the isolated proteins. Thus, phase-separation of proteins did not limit intermolecular disulfide formation. Pretreatment of proteins with aqueous ethanol did not substantially influence their subsequent polymerization during prolonged heating in water. However, ethanol pretreatment of gluten impacted heat-induced polymerization of BSA in gluten-BSA mixtures.

Key words

Differential scanning calorimetry, size exclusion, polymerization, ethanol pretreatment

Chemical compounds studied in this article

Water (PubChem CID: 962); Ethanol (PubChem CID: 702); Sodium dodecyl sulfate (PubChem CID: 342365); Dithiothreitol (PubChem CID: 19001); Cysteine (PubChem CID: 5862); 5,5'-dithiobis(2-nitrobenzoic acid) (PubChem CID: 6254); Urea (PubChem CID: 1176); Tetrasodium ethylenediaminetetraacetate (PubChem CID: 6144); Lanthionine (PubChem CID: 102950); Lysinoalanine (PubChem CID: 29269)

1. Introduction

A key concept in protein science is that form and function are inseparable. Protein aggregation is related to neurodegenerative diseases (Chiti & Dobson, 2006) and sickle cell anemia (De Llano & Manning, 1994), but also to texture and structural properties of food products (Singh, 1991; Totosaus, Montejano, Salazar, & Guerrero, 2002). For instance, polymerization of wheat gluten proteins positively impacts on bread quality (Lagrain, Thewissen, Brijs, & Delcour, 2008). Similarly, the formation of a strong gluten network, mainly through disulfide (SS) bond formation and reshuffling, is crucial for the quality of pasta, some cookie and other cereal-based products (Delcour et al., 2012). Heat-induced denaturation of globular proteins, *i.e.* the transformation of the native to a disordered state, changes protein functionality and often induces gelling. Such aggregation involves both non-covalent interactions and covalent cross-links, the latter mainly SS bonds (Foegeding & Davis, 2011; Mine, 1995). In contrast to albumins and globulins, wheat gluten proteins are soluble neither in water nor in aqueous salt solutions (Osborne, 1907). Also, they do not show endothermic denaturation peaks when analyzed by differential scanning calorimetry (DSC) (Erdogdu, Czuchajowska, & Pomeranz, 1995). In *inter alia* cake and egg noodles, wheat gluten and globular proteins co-exist. Co-protein effects due to interactions and reactions between different protein types can impact such food systems (Erickson, Campanella, & Hamaker, 2012; Rombouts, Lagrain, & Delcour, 2012). The influence of different types of proteins on each other's behavior remains to be further investigated.

Protein aggregation in aqueous salt solutions (*e.g.* physiological conditions) has received much attention because of its importance and relevance in life science and food systems. Alcohols are used in products ranging from food additives to preservatives in cosmetics (Nair, 2001) and can impact the stability and folding of proteins (Thomas & Dill, 1993). In food and food system related applications, aqueous ethanol is *e.g.* used for precipitating proteins from cheese whey, purifying protein from soy

flakes (Hua, Huang, Qiu, & Liu, 2005; Morr & Lin, 1970) and for marinating meat or fish with beer or wine (Melo, Viegas, Petisca, Pinho, & Ferreira, 2008). Furthermore, some edible films based on soy proteins or wheat gluten are prepared in the presence of aqueous ethanol (Ali, Ghorpade, & Hanna, 1997; Gontard, Guilbert, & Cuq, 1992). Instead of using water to produce vital wheat gluten in a traditional Martin process (Van Der Borght, Goesaert, Veraverbeke, & Delcour, 2005), an energy and water saving method uses (aqueous) ethanol as washout liquid at low temperature (Robertson & Cao, 1998). It results in vital wheat gluten which has better dough mixing properties than the water-equivalent (Robertson & Cao, 2002). Moreover, ethanol pretreatment changes the rheological properties of wheat flour (Robertson et al., 2011). Even at low levels, similar to those in fermenting bread dough, ethanol decreases dough extensibility and makes dough more stiff and tenacious (Jayaram et al., 2014). Notwithstanding the above, the impact of aqueous alcohols on (heat-induced) aggregation of wheat gluten proteins remains to be studied.

Interactions between amino acid side chains and their immediate environment affect protein aggregation. Small changes in temperature, pH, ionic strength and polarity can impact the conformation of globular proteins. While native and denatured proteins do not aggregate easily in aqueous environments due to buried hydrophobic regions, partially unfolded proteins with notable secondary structure are more prone to aggregate (Chi, Krishnan, Randolph, & Carpenter, 2003). Alcohols are less polar than water and thus weaken hydrophobic interactions and enhance polar interactions thereby facilitating protein denaturation (Thomas & Dill, 1993). Often, proteins denature in aqueous-organic media but not in the corresponding pure organic solvent (Griebenow & Klibanov, 1996). Furthermore, especially the larger alkyl alcohols stabilize α-helical conformations of unfolded proteins (Hirota, Mizuno, & Goto, 1997). Because aqueous alcohols partially unfold proteins, they can induce protein aggregation (Singh, Cabello-Villegas, Hutchings, & Mallela, 2010). With increasing ethanol concentration, bovine serum albumin (BSA), a protein of milk, whey and meat (Belitz, Grosch, & Schieberle, 2009), tends to lose its secondary structure and form aggregates (Liu et al., 2010). Similarly, with increasing alcohol concentrations, partial and progressive dehydration and alcohol binding transforms gel-like sediments of milk and soy proteins into opaque flocks (precipitates) (Boulet, Britten, & Lamarche, 2001). It is clear neither whether alcohol-induced aggregation of albumins and globulins in the above examples is due to non-covalent interactions or covalent cross-links, nor whether and how alcohols would influence heat-induced aggregation.

While food systems often contain more than one protein type, protein denaturation and polymerization have mainly been studied in single protein systems. Due to differences in solubility, proteins in complex food systems can be present in various phases. In this context, Polyakov *et al.* (1997) used the term protein thermodynamic incompatibility. They even stated that differences in

hydrophilic character between various protein types trigger phase separation and thereby promote interactions between proteins with similar conformation (Polyakov et al., 1997). Given the impact of alcohols on protein conformation and solubility, it is of interest to compare heat-induced polymerization of complex systems in water to that in aqueous ethanol.

Against this background, structural changes during heat treatment of various proteins in aqueous ethanol were compared to those in water. BSA and glycinin, one of the two most abundant soy proteins (Liu et al., 2007) were chosen as model globular proteins. Gliadin, the monomeric protein fraction of wheat gluten consists of α-, γ- and ω-gliadin. It was selected as model prolamin (Wieser, 2007). First, the impact of water and aqueous ethanol on denaturation and covalent network formation of isolated proteins was studied. In addition, sulfhydryl (SH) oxidation, SH-SS interchange reactions and β-elimination reactions were investigated. The second aim was to evaluate the impact of different protein types on each other's denaturation and polymerization during heating in water and aqueous ethanol. Here, the importance of protein incompatibility during heat treatment of complex systems containing different protein types was investigated. Furthermore, the impact of a pretreatment or isolation with aqueous ethanol of proteins was studied on the polymerization behavior of isolated proteins and mixtures thereof.

2. Materials and methods

2.1 Materials

Gluten [83.2% protein, on dry matter (dm) basis] from wheat (cultivar Paragon, RAGT, Ickleton, United Kingdom) and soy glycinin (98.1% dm protein) from soy flour (L.I. Frank, Twello, The Netherlands) were isolated as in Lambrecht *et al.* (2015). Gliadin was extracted from gluten (20.0 g) with 70% (v/v) ethanol (250 ml). After centrifugation (10 000 *g*, 10 min), ethanol was evaporated (Rotavapor R3000, Büchi, Flawil, Switzerland) from the supernatant. Gliadin (87.7% dm protein) was freeze dried, ground in a laboratory mill (IKA, Staufen, Germany), and passed through a 250 µm sieve. BSA (fraction V for biochemistry, 98.2% dm protein) was from Acros Organics (Geel, Belgium). All chemicals were at least of analytical grade and from Sigma-Aldrich (Steinheim, Germany) unless specified otherwise. Dithiothreitol (DTT), disodium hydrogen phosphate and sodium dihydrogen phosphate were from VWR International (Leuven, Belgium).

2.2 Protein content

Protein content was determined in triplicate, using an adaptation of AOAC Official Method 990.03 (AOAC, 1995), with an automated Dumas protein analysis system (EAS Variomax N/CN, Elt, Gouda,

The Netherlands). Conversion factors (5.7 for gluten and gliadin; 6.25 for soy glycinin and BSA) were used to calculate protein from nitrogen contents.

2.3 Aqueous ethanol pretreatment

BSA, soy glycinin and gluten (500.0 mg dm protein) were shaken for 60 min with 5.0 ml 50% (v/v) ethanol. Gluten (500.0 mg dm protein) was also pretreated with 70% (v/v) ethanol (5.0 ml) in a similar way to simulate conditions during gliadin isolation. Ethanol was evaporated from samples using a Rotational Vacuum Concentrator (Q-lab, Vilvoorde, Belgium, 35 °C, 1.0 mbar). Aqueous ethanol pretreated (EtPT) samples were freeze-dried and ground using a mortar and pestle.

2.4 Heat treatment

Deionized water, 10% or 50% (v/v) ethanol (5.0 ml) were added to BSA, soy glycinin or gliadin (500.0 mg or 166.7 mg dm protein). Furthermore, blends of either gluten or gliadin with BSA or soy glycinin [500.0 mg or 166.7 mg protein in total, ratio (2:1)] were mixed with 5.0 ml deionized water or 50% (v/v) ethanol. EtPT proteins and mixtures thereof were suspended in water. The reaction tubes (glass, inner diameter = 27 mm, outer diameter = 34 mm, height = 100 mm) were hermetically sealed and horizontally shaken at 100 °C for 6, 60 or 120 min. Heat-treated samples were immediately cooled in water. Unheated samples were shaken for 60 min at room temperature. Samples were considered colloidally stable if their proteins in a stirred reaction mixture did not precipitate after three days of standing at room temperature. Ethanol was evaporated from samples using a Rotational Vacuum Concentrator (35 °C, 1.0 mbar). All samples were freeze-dried and ground using a mortar and pestle.

2.5 Determination of protein extractability and molecular weight distribution

SE-HPLC was conducted as in Lambrecht *et al.* (2015) using a LC-2010 system (Shimadzu, Kyoto, Japan) with automated injection. To extract proteins under non-reducing conditions, samples (1.0 mg protein) were shaken (60 min, room temperature) with 1.0 ml 0.050 M sodium phosphate buffer (pH 6.8) containing 2.0% (w/v) SDS. Proteins were also extracted under reducing conditions, *i.e.* under nitrogen atmosphere using the same buffer containing 1.0% (w/v) DTT. All extractions were in triplicate. After centrifugation (10 000 *g*, 10 min) and filtration (Millex-HP, 0.45 µm, polyethersulfone; Millipore, Carrigtwohill, Ireland) extracts (20 µl) were loaded on a Biosep-SEC-S4000 column (Phenomenex, Torrance, CA, USA). The eluent was 0.050 M sodium phosphate buffer (pH 6.8) containing 2.0% (w/v) SDS (flow rate 1.0 ml/min, 30 °C). Protein elution was monitored at 214 nm. The level of protein extractable in SDS-containing media (SDS-EP) under non-reducing conditions was calculated from the corresponding peak area and expressed as a percentage of the

assumed total area. The assumed total area of a sample was that of the corresponding unheated sample extracted under reducing conditions. SDS-extractable polymeric compounds were collected, reduced with DTT (room temperature, 60 min) and again separated using SE-HPLC.

2.6 Differential scanning calorimetry

Protein denaturation properties were determined at least in triplicate with a Q2000 DSC (TA instruments, New Castle, DE, USA) as described by Dries *et al.* (2014). Samples were accurately weighed (2.20-4.00 mg) in an aluminum pan (Perkin-Elmer, Waltham, MA, USA) and high pressure steel pans (Mettler-Toledo, Zaventem, Belgium) when analyzed in deionized water on the one hand and in aqueous ethanol [10% or 50% (v/v) ethanol] on the other. Pans [1/3 (w/w) protein/solvent] were hermetically sealed and heated from 0 °C to 120 °C at 4 °C/min. Empty pans were used as reference. Calibration was with indium. The denaturation onset, peak, conclusion temperatures, temperature ranges and enthalpies were determined using Universal Analysis 2000 software (TA Instruments).

2.7 Determination of free sulfhydryl content

Free SH groups were determined colorimetrically with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). Samples (1.0-1.5 mg protein) were shaken (10 min, room temperature) with 200 µl water or 50% (v/v) ethanol. Afterwards, 800 µl sample buffer [0.050 M sodium phosphate buffer (pH 6.5) containing 2.0% (w/v) SDS, 3.0 M urea and 1.0 mM tetrasodium ethylenediaminetetraacetate] and 100 µl DTNB reagent [0.1% (w/v) in sample buffer] were added and the samples were shaken. The absorbance at 412 nm was read exactly 10 min after adding DTNB reagent. Absorbance values were converted to concentrations of free SH using a calibration curve with reduced glutathione. Controls without DTNB or sample were used to correct for background absorbance of DTNB and sample. All analyses were performed in triplicate.

2.8 Determination of oxidation reaction rate

DTT (0.05 mg/ml) was dissolved in water, 10% or 50% (v/v) ethanol and heated for several hours at 100 °C. After cooling on ice (5 min), sample (200 µl) was added to 800 µl sample buffer (as described in section 2.7) and 100 µl DTNB [0.1% (w/v) in sample buffer] reagent. After exactly 10 min, the absorbance at 412 nm was read. All analyses were performed in triplicate.

2.9 Determination of sulfhydryl-disulfide exchange reaction rate

Rates of SH (DTT)-SS (DTNB) exchange reactions were monitored in triplicate under nitrogen atmosphere. To 900 µl DTT [0.25% (w/v) DTT in 1.0% (w/v) SDS with 0.83 M urea], 100 µl DTNB [0.1%

(w/v) in 1.0% SDS (w/v) with 0.83 M urea] and 1000 µl solvent [1.0% (w/v) SDS in water and/or 1.0% SDS (w/v) in ethanol] were added to a final concentration of 0%, 10% and 50% (v/v) ethanol. The absorbance readings at 412 nm were monitored for 10 min at room temperature. The absorbance of the first measurements were used to correct for background absorbance. Results were corrected for the difference in extinction coefficient of DTNB in aqueous ethanol compared with water.

2.10 Analysis of lysinoalanine and lanthionine cross-links

The dehydroalanine-derived cross-links lanthionine and lysinoalanine were quantified in gliadin after heating (100.0 mg dm protein/ml solvent) in water or 50% (v/v) ethanol for 15 hours at 130 °C. After ethanol evaporation (Rotational Vacuum Concentrator, 35 °C, 1.0 mbar), samples were freeze-dried and ground. (Iso)peptide bonds were hydrolyzed by heating at 110 °C for 24 h in 6.0 M HCl and (cross-linked) amino acids were separated and quantified using high-performance anion-exchange chromatography with pulsed amperometric detection as in Rombouts *et al.* (2009).

2.11 Lab-on-a-Chip capillary electrophoresis

Soy glycinin in 50% (v/v) ethanol (20.0 mg protein/ml) was heated for 5 min at 70 °C. After centrifugation (10 000 *g*, 10 min) supernatant and precipitate were separated and freeze-dried. To these samples and isolated glycinin (7.0 mg protein), 1.0 ml sodium phosphate buffer (0.050 M; pH 6.8) containing 2.0% (w/v) SDS was added and samples were shaken (30 min, room temperature). After centrifugation (13 000 *g*, 10 min), supernatant (8 µl) was mixed with Agilent sample buffer (4 µl, Agilent Technologies, Santa Clara, CA, USA) and heated at 100 °C for 5 min. After cooling, 168 µl deionized water was added. The mixture (6 µl) and molecular weight markers were applied on an Agilent LabChip of a protein 230 kit and analyzed with an Agilent 2100 Bioanalyzer system.

2.12 Circular dichroism

Circular dichroism (CD) spectra of (EtPT) BSA in water and 50% (v/v) ethanol were recorded in the far-UV range (190 to 250 nm) at room temperature with a Jasco J-810 Spectropolarimeter (Jasco Benelux, Maarssen, The Netherlands) using a quartz cell with 1.0 mm path length and a protein concentration of 0.1 mg/ml. Data were expressed as molar residual ellipticity [Θ], defined as

$$\Theta = \frac{100\,[\theta]_{obs}}{\mathrm{l\,c}}$$

were $[\Theta]_{\mathrm{obs}}$ is the observed molar ellipticity in degrees, l the length of the cell light path in centimeters and c the protein concentration in mol/l.

2.13 Statistics

Significant differences ($\alpha < 0.05$), based on at least three individual measurements, were determined with the one-way ANOVA procedure using JMP® Pro 11.2.0 (SAS Institute, Cary, NC, USA). Corresponding Tukey grouping coefficients are given.

3. Results and discussion

3.1 Impact of water and aqueous ethanol on protein network formation

After shaking for 60 min at room temperature in water or 10% (v/v) ethanol, freeze-drying and subsequently extracting with SDS-containing media, both monomeric and dimeric BSA were found (Figure 1.1). In comparison, BSA in 50% (v/v) ethanol yielded, beside monomers and dimers, both extractable and non-extractable higher molecular weight compounds. Water and 10% (v/v) ethanol had no impact on the SDS-EP content of unheated BSA (100% ± 7%) while 50% (v/v) ethanol reduced it to 68% ± 6%. Under reducing conditions, all BSA samples were fully extractable in SDS-containing buffer and only consisted of monomers and dimers (results not shown). The above demonstrate that SS cross-links were formed between BSA molecules at 50% (v/v) ethanol already at room temperature. Liu *et al.* (2010) reported the formation of larger (100 nm) BSA aggregates in 50% (v/v) ethanol. Our results demonstrate that not only non-covalent interactions but also covalent cross-links contribute to such aggregates. Liu *et al.* (2010) reported that the helicity of BSA decreased when the ethanol concentration increased from 10% (v/v) until complete unfolding was observed at 50% (v/v) ethanol. In agreement, DSC analyses of BSA (Figure 2.A) showed that the denaturation temperatures and enthalpies decreased with increasing ethanol concentration until complete denaturation at 50% (v/v) ethanol. It is reasonable to assume that the unfolding induced by aqueous ethanol facilitates SS cross-linking. At room temperature, SH-SS exchange reactions occurred faster with decreasing ethanol concentration (Figure 3). In addition, more free SH groups of BSA were accessible in 10% (5.1 ± 0.9 µmol SH/g protein) and 50% (v/v) ethanol (4.5 ± 0.4 µmol SH/g protein) than in water (2.5 ± 0.3 µmol SH/g protein). Furthermore, to polymerize, proteins have to overcome an energy barrier mainly formed by electrostatic and van der Waals interactions. The immediate environment of protein impacts its colloidal stability and thus its tendency to precipitate (Chi et al., 2003). At room temperature, BSA (100.0 mg protein/ml) was colloidally stable in water and 10% (v/v) ethanol but not in 50% (v/v) ethanol in which it also polymerized. Heating BSA (100.0 mg protein/ml) in either water, 10% or 50% (v/v) ethanol rapidly reduced its SDS-EP content to 3% ± 1% (Figure 1.1). At a lower concentration (33.3 mg protein/ml), BSA was colloidally stable and fully extractable in SDS containing medium at room temperature in both water and 50% (v/v) ethanol. However, more SDS-extractable polymers were formed in 50% (v/v) ethanol than in water (Figure 4).

Gliadin, shaken for 60 min at room temperature in water or 10% (v/v) ethanol and subsequently extracted in SDS-containing medium, eluted over a wide range in SE-HPLC. The largest gliadins (ω-gliadins) eluted between 8 min 20 s and 8 min 55 s, while most α- and γ- gliadins eluted between 8 min 42 s and 9 min and 36 s. While 10% (v/v) ethanol had significant impact neither on the molecular weight nor on the extractability of gliadin in SDS-containing media at room temperature (Figure 1.2), it substantially reduced heat-induced polymerization compared to water as solvent. In 50% (v/v) ethanol, unheated gliadin was colloidally stable and the extractability of gliadin even remained constant during heating at 100 °C (Figure 1.2). SH oxidation occurs faster with decreasing ethanol concentration during heating at 100 °C (Figure 5). Gliadin lacks free SH groups. Its polymerization in water is initiated by β-elimination reactions from intramolecular SS bonds in α- and γ-gliadins which form dehydroalanine and free SH groups (Rombouts, Lagrain, Brijs, & Delcour, 2010). The lack of polymerization of gliadin in 50% (v/v) ethanol indicate that the extent of β-elimination reactions is limited. However, lanthionine, the product from Michael addition of the SH group from cysteine to dehydroalanine, was detected after severe and prolonged heating (15 hours, in water or 50% (v/v) ethanol at 130 °C) of gliadin. This indicates that severe heating in 50% (v/v) ethanol was still able to induce β-elimination reactions. ω-Gliadins, which lack SS bonds, remained extractable during heating in SDS-containing medium in either solvent. The slight heat-induced reduction of this peak is due to the polymerization of some α- and γ-gliadins co-eluting with ω-gliadins.

With increasing ethanol concentration, the level of SDS-extractable soy glycinin polymers (before 7 min 48 s) at room temperature increased (Figure 1.3), which was ascribed to increasing SH-SS exchange reaction rates (Figure 3). Glycinin contains *ca.* 20 SS bonds which are mostly buried in the interior of the protein, and no free SH groups (Draper & Catsimpoolas, 1978). Sodium bisulfite reduced some of these SS bonds into free SH groups during the isolation protocol. However, no significant differences between the levels of accessible SH groups in water (1.7 ± 0.3 μmol Cys/g protein) and in 50% (v/v) ethanol (1.8 ± 0.2 μmol Cys/g protein) were noted. With increasing ethanol concentrations, the extractability loss during prolonged (> 6 min) heating decreased (Figure 1.3). Heating glycinin at 100 °C in 50%, 10% (v/v) ethanol or water reduced the SDS-EP content to 73% (± 2%), 43% (± 3%) or 37% (± 2%) after 60 min and to 65% (± 5%), 40% (± 2%) or 33% (± 1%) after 120 min, respectively. Also, SH oxidation, SH-SS exchange reactions and β-elimination reactions occurred faster with decreasing ethanol concentration (Figures 3, 5 and 1.2). Furthermore, soy glycinin proteins precipitated in water and 10% (v/v) ethanol while they were stable in 50% (v/v) ethanol. Ethanol impacted not only the covalent cross-linking, but also the denaturation of soy glycinin. Glycinin denatured in water with a peak temperature of 96.8 °C ± 0.1 °C and in 10% (v/v) ethanol with a peak temperature of 81.4 °C ± 0.1 °C (Figure 2.A). In 50% (v/v) ethanol, glycinin showed two

endothermic DSC peaks and the total denaturation enthalpy was lower than that in water or 10% (v/v) ethanol (Figure 2.A). It was investigated whether those two peaks correspond to basic (*ca.* 20 kDa) and acidic (*ca.* 38 kDa) polypeptides. In water, glycinin consists of subunits, each containing a basic and an acidic polypeptide connected by an SS bond (except for the acidic polypeptide A_4) (Staswick, Hermodson, & Nielsen, 1984) which is cleaved upon heating (Hashizume & Watanabe, 1979). However, heating glycinin to 70 °C in 50% (v/v) ethanol produced supernatants and precipitates which both contained acidic and basic polypeptides, but also glycinin subunits (results not shown). As discussed for gliadin, β-elimination reactions only occur to a limited extent in 50% (v/v) ethanol. So, it is very unlikely that the two distinct denaturation peaks are due to cleavage of the SS bonds between acidic and basic polypeptides. Instead, it is hypothesized that in 50% (v/v) ethanol both hexameric and trimeric glycinin complexes exist. The latter denature at a lower temperature than the former (Lakemond, de Jongh, Hessing, Gruppen, & Voragen, 2000). No trimeric nor hexameric complexes were present in the unheated glycinin extract due to the presence of SDS. Soy glycinin monomers eluted between 7 min 48 s and 9 min (Figure 1.3). Acidic and basic polypeptides were detected after 9 min. Protein compounds eluting before 7 min 48 s were attributed to polymerization during shaking in the selected solvent.

3.2 Network formation between different proteins in water and aqueous ethanol

In water, unheated BSA was soluble while unheated gliadin precipitated. The SDS-EP values of all BSA-gliadin mixtures (ratio 1:2) heated in water at 100 °C were lower than expected based on the extractability losses of the isolated proteins (Figure 6.1A). Thus, BSA and gliadin impacted each other's polymerization. SE-HPLC showed that after 6 min of heating, a small peak containing SDS-extractable polymers eluted between 5 and 6 min which did not appear in the profiles of the (heated) isolated proteins (results not shown). These polymeric compounds consisted of both BSA and gliadin. That, in contrast, 6 min heating of isolated BSA already resulted in complete extractability loss, allowed concluding that gliadin slows down BSA polymerization. In support, addition of gliadin increased the denaturation temperature of BSA in water from 62.0 °C ± 0.4 °C (Figure 2.A) to 83.1 °C ± 0.2 °C (Figure 2.B). Rombouts *et al.* (2012) already described that, under the conditions they used, gluten increases the denaturation temperature of BSA at pH 8.0 to *ca.* 83.7 °C. Apparently, gliadin can also stabilize BSA. As expected, after 60 min of heating, BSA was mostly incorporated in the protein network. Here, the difference between measured and expected SDS-EP levels was due to substantial gliadin polymerization (Figure 6.1A, peak II). BSA facilitated the incorporation of gliadin in the protein network through SH-SS interchange reactions. A small peak of protein eluting at *ca.* 8 min 40 sec remained after 120 min of heating, and corresponds to ω-gliadin.

In 50% (v/v) ethanol, both BSA and gliadin were colloidally stable at room temperature. The total SDS-EP values of BSA-gliadin mixtures during prolonged (> 6 min) heating in 50% (v/v) ethanol were as expected based on the SDS-EP values of the isolated proteins (Figure 6.1B). At room temperature, BSA consisted of monomers, dimers and a wide range of (SDS-extractable) polymers in 50% (v/v) ethanol (Figure 1.1C). After 60 min of heating, little if any BSA monomer remained (peak overlap at *ca.* 8 min 40 sec with ω-gliadin) and gliadin ceased polymerizing (Figure 6.1B). The SDS-EP content of the BSA-gliadin mixture reached a plateau at *ca.* 66%.

In water, both soy glycinin and gliadin precipitated at room temperature. After heating in water, less protein was extracted in SDS-containing medium from a glycinin-gliadin mixture than from the isolated proteins (Figure 6.2A). Especially gliadin polymerized faster and to a larger extent with glycinin than alone. Addition of gliadin to glycinin (weight ratio 2:1) slightly decreased the denaturation temperature of glycinin in water, but it did not change the associated enthalpy (Figure 2.B). Thus, when heated in water, glycinin impacted gliadin more than vice versa.

In 50% (v/v) ethanol, both unheated soy glycinin and gliadin were colloidally stable. After heating in 50% (v/v) ethanol, the glycinin-gliadin mixture had higher extractability than expected based on the extractability losses of the isolated proteins (Figure 6.2B). Both with or without glycinin, gliadin (Figure 6.2B, Peak II) remained fully extractable during heating in 50% (v/v) ethanol. In contrast, the extractability of glycinin (Figure 6.2B, Peak III) decreased less in the presence than in the absence of gliadin, in line with the impact of gliadin on the denaturation of glycinin in 50% (v/v) ethanol. In absence of gliadin, trimeric and hexameric glycinin complexes denatured at 54.5 °C and 92.5 °C, respectively, as stated in section 3.1 (Figure 2.A). In the presence of gliadin, all glycinin denatured at 54.5 °C, probably as trimeric complexes (Figure 2.B). It is hypothesized that the hexameric complexes, present in water with and without gliadin and to a small extent in 50% (v/v) ethanol without gliadin, but absent in 50% (v/v) ethanol with gliadin, contribute significantly to protein network formation. Thus, when heated in 50% (v/v) ethanol gliadin impacted glycinin more than vice versa.

Polyakov *et al.* (1997) described protein solubility as a key factor determining protein compatibility. Applying their theory to the present case, protein interactions between BSA and gliadin would be favored in 50% (v/v) ethanol, where both protein types are soluble, but not in water, where BSA and gliadin phase-separate. However, this work showed that both protein mixtures polymerize to a larger extent in water than in 50% (v/v) ethanol. The limited β-elimination, decreased SH oxidation and SH-SS exchange reaction rate, conformational changes in 50% (v/v) ethanol and the colloidal stability of proteins in the same medium, reduced covalent cross-linking. Moreover, in water, the proteins in

both mixtures polymerized to a larger extent than expected based on the extractability losses of the isolated proteins, while in 50% (v/v) ethanol they polymerized equally or less than expected. Thus, a co-protein effect occurs in water and not in 50% (v/v) ethanol where both protein types are soluble.

3.3 Impact of aqueous ethanol pretreatment on protein network formation

We here evaluated the impact of the use of aqueous ethanol to isolate proteins, on their subsequent network formation and denaturation in water. At room temperature, EtPT did not affect the SDS-EP content of BSA in water (Table 1). Thus, while SS cross-links reduced the SDS-EP of BSA in 50% (v/v) ethanol to 68% (±6%) at room temperature (Section 3.1), subsequent drying and suspension of the sample in water restored the SDS-EP content, due to SH-SS exchange reactions which released BSA monomers from the protein network. Much as in 50% (v/v) ethanol, polymers, dimers and monomers extractable in SDS containing medium were present in the unheated sample (profile not shown). However, EtPT slightly slowed down the extractability loss during heating, demonstrated by a higher SDS-EP content of BSA with than without EtPT after 6 min of heating (Table 1). Similar extents of polymerization were obtained with or without EtPT upon prolonged heating at 100 °C. EtPT also affected BSA denaturation (Figure 2.C). After denaturation in 50% (v/v) ethanol, suspension in water partly reversed denaturation. It yielded BSA which denatured at higher temperatures but with lower enthalpy. CD analyses also showed clear differences between the conformation of BSA in water and in 50% (v/v) ethanol, while that after EtPT was intermediate between both (Figure 7).

At room temperature, EtPT of soy glycinin increased the level of SDS-extractable high molecular weight compounds (SE-profile not shown). After 6 min of heating, polymerization of soy glycinin was slightly slowed down after EtPT. Prolonged (> 6 min) heating in water did not impact the overall SDS-EP content (Table 1). While soy glycinin unfolded into trimeric and hexameric complexes in 50% (v/v) ethanol (section 3.2), DSC analyses of EtPT soy glycinin in water showed only one single peak temperature (95.8 °C ± 0.4 °C) suggesting that the more thermostable hexameric conformation was again favored in water. However, EtPT of glycinin reduced the enthalpy and slightly decreased the denaturation temperature (Figure 2.C).

Moreover, EtPT impacted neither the molecular weight distribution nor the SDS-EP content of gluten before or after prolonged (> 6 min) heating at 100 °C in water (Table 1). After 6 min of heating, polymerization of gluten was slightly increased as a result of EtPt. In conclusion, EtPT altered the conformation, denaturation and heat-induced polymerization at short times (≤ 6 min) of BSA and soy glycinin, but did not significantly impact the polymerization of proteins during prolonged heating in water at 100 °C.

379 Furthermore, it was investigated whether pretreatment of one protein type with aqueous ethanol
380 affected its co-polymerization with other protein types. Gluten addition increased the denaturation
381 temperature and enthalpy of BSA after EtPT (Figure 2.C). However, the stabilizing effect during heat-
382 induced denaturation of gluten on BSA after EtPT was less than gliadin had on BSA without EtPT
383 (Figure 2.B). However, EtPT of BSA did not impact the overall extractability loss of a BSA-gluten
384 mixture during heating in water (Table 2).

385 In contrast, pretreatment of gluten with 50% (v/v) ethanol increased the extractability loss of a BSA-
386 gluten mixture during heating in water (Table 2). EtPT of gluten increased gliadin incorporation in the
387 protein network (SE-profiles not shown). While the presence of BSA increased the extent of
388 polymerization of gluten, gluten slowed down the extent of polymerization of BSA, especially after
389 EtPT of gluten. After short heating times (2 min), SDS-extractable polymers eluting at 5 min 40 sec
390 contained both BSA and gluten protein and pretreatment of gluten with 70% (v/v) ethanol increased
391 their relative levels (Figure 8). Possible explanations for the increased extractability loss of a BSA-
392 gluten mixture after EtPT of gluten include (i) conformational changes of gluten proteins, (ii)
393 redistribution of lipids and (iii) release of gliadin from the glutenin network. Changes in glutenin
394 secondary structure due to heating have been related to changes in gluten physicochemical
395 properties (hydrophobicity, SH- and SS content) (Weegels, de Groot, Verhoek, & Hamer, 1994).
396 Glycolipids are preferentially associated with glutenin while phospholipids tend to interact with
397 gliadin and lipid binding proteins in gluten (McCann, Small, Batey, Wrigley, & Day, 2009). The
398 extraction of bound lipids with aqueous ethanol may redistribute lipids and thereby change the
399 interactions between proteins. The EtPT of gluten also releases gliadin from the glutenin network,
400 thereby increasing its opportunity to react with BSA.

401 ## 4. Conclusion

402 Already at room temperature, contact with ethanol impacts the molecular weight distribution of
403 some proteins. For instance, BSA aggregated in 50% (v/v) ethanol, not merely due to non-covalent
404 interactions but also due to SS cross-linking. This paper also demonstrated, for the first time, the
405 impact of aqueous ethanol on heat-induced cross-linking of gliadin and glycinin. Increasing the
406 ethanol concentration reduced polymerization of soy glycinin and gliadin at 100 °C. Gliadin
407 polymerization was even blocked in 50% (v/v) ethanol. Aqueous ethanol decreased the rate of β-
408 elimination, SH oxidation and SH-SS exchange reactions. Furthermore, it altered the colloidal
409 stability, protein conformation and availability of reactive groups. In water but not in aqueous
410 ethanol, gliadin-BSA and gliadin-soy glycinin mixtures polymerize more than expected based on
411 polymerization of the isolated proteins under equal conditions. An important finding is that

thermodynamic compatibility is not the key parameter enhancing covalent network formation between proteins. In complex aqueous food systems with or without ethanol, different protein types influence each other's polymerization, even when they are phase-separated. Pretreatment with aqueous ethanol altered protein conformations and denaturation properties but did not influence network formation during prolonged heating of isolated proteins. In contrast, pretreatment of gluten impacted heat-induced polymerization of BSA in gluten-BSA mixtures. Increased knowledge on ethanol-induced protein modifications, both in terms of conformation and functionality, is not only helpful to better understand existing food systems, it also opens perspectives for exploring new processing steps towards enhanced protein functionalities.

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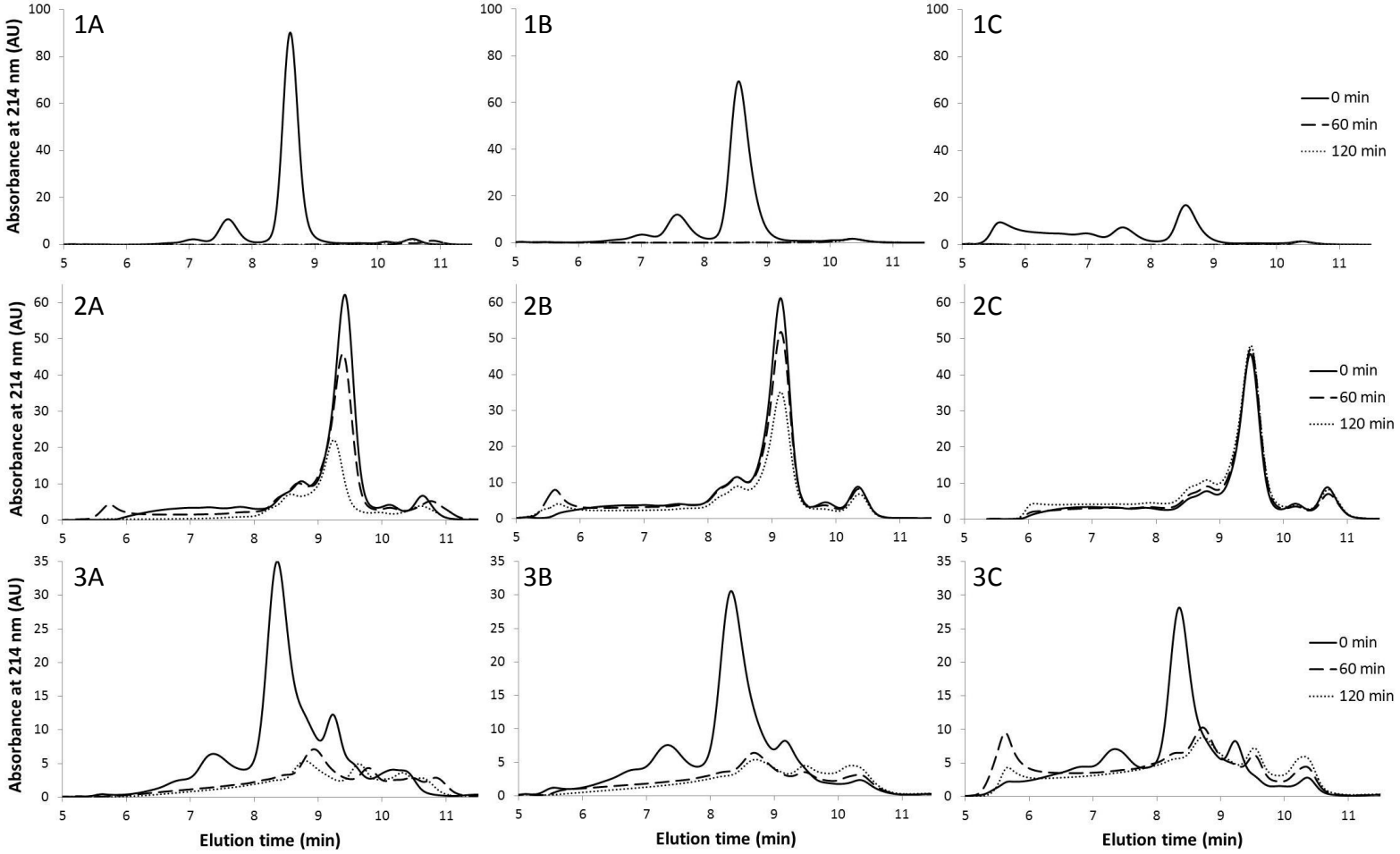
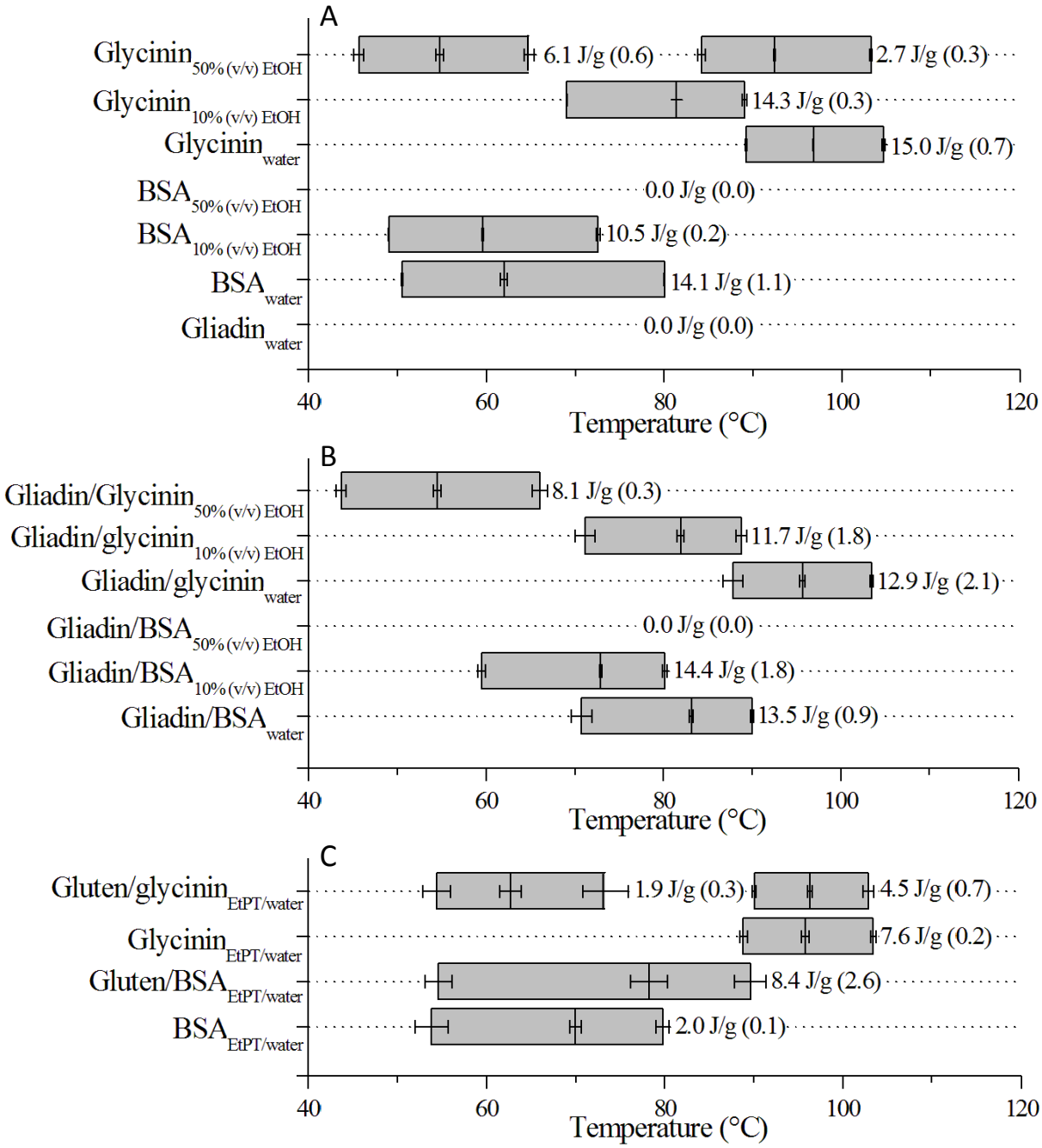


Figure 1. SE-HPLC profile of protein extracts in sodium dodecyl sulfate containing medium (SDS-EP) of bovine serum albumin (BSA, 1), gliadin (2) and soy glycinin (3) before and after heat treatment at 100 °C for 60 and 120 min in water (A), 10% (v/v) ethanol (B) and 50% (v/v) ethanol (C). During heating a concentration of 100.0 mg protein/ml solvent A, B or C was used. AU, arbitrary units.



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549 **Figure 2.** Schematic overview of the denaturation temperature ranges and enthalpic transition data
550 of bovine serum albumin (BSA), soy glycinin (A) and mixtures thereof with gliadin or gluten (ratio 1:2,
551 B) in water, 50% and 10% (v/v) ethanol (EtOH) and in water after pretreatment with 50% (v/v)
552 ethanol (EtPT, C). Start and end points of the bars represent the denaturation onset and conclusion
553 temperatures. The vertical lines inside the bars are the denaturation peak temperatures. The
554 enthalpy data are expressed on the amount of globular proteins in the sample. Standard deviations
555 are given between brackets.

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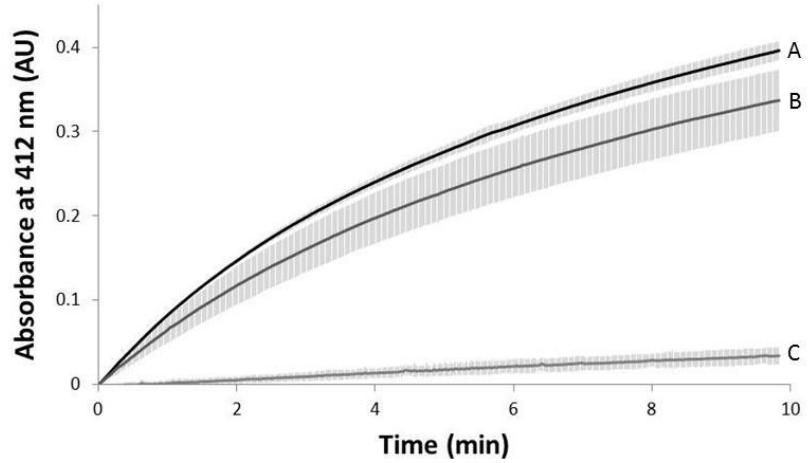


Figure 3. Absorbance measurement in time of the sulfhydryl-disulfide exchange reaction between dithiothreitol and 5,5'-dithiobis(2-nitrobenzoic acid) in water (A), 10% (v/v) ethanol (B) and 50% (v/v) ethanol (C). AU, arbitrary units. Standard deviations are given as error bars.

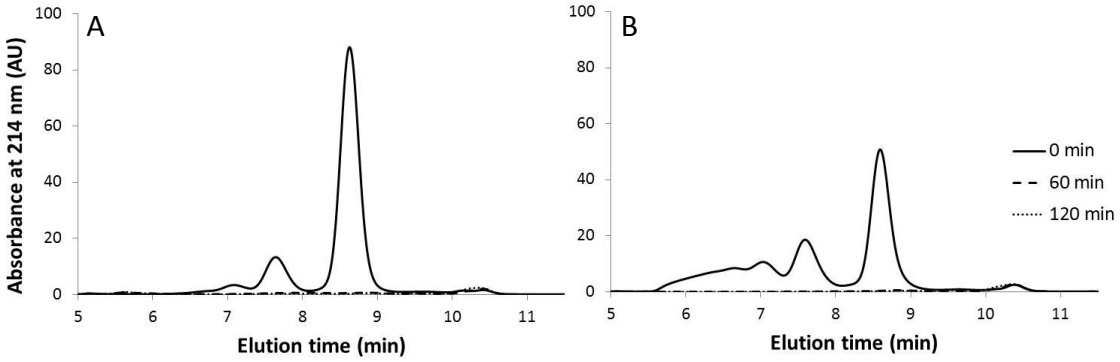


Figure 4. SE-HPLC profile of protein extracts in sodium dodecyl sulfate containing medium (SDS-EP) of bovine serum albumin (BSA) heated in water (A) and 50% (v/v) ethanol (B) at a concentration of 33.3 mg protein/ml solvent. AU, arbitrary units.

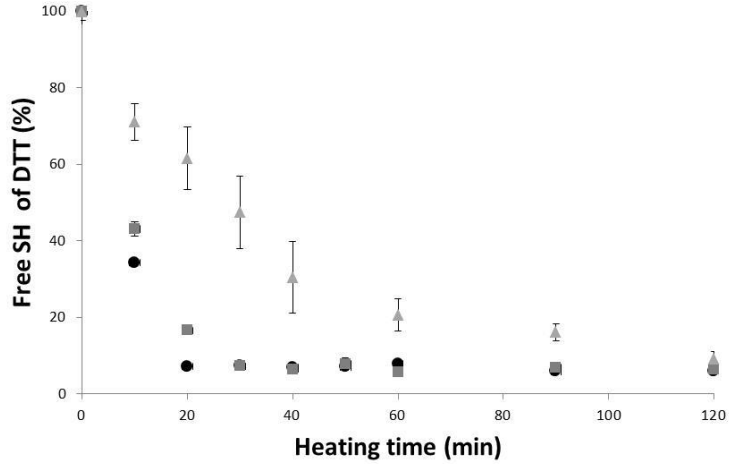


Figure 5. Decrease in free sulfhydryl (SH) content of dithiothreitol (DTT) heated at 100 °C in water (○), 10% (v/v) ethanol (□) or 50% (v/v) ethanol (△). Standard deviations are given as error bars.

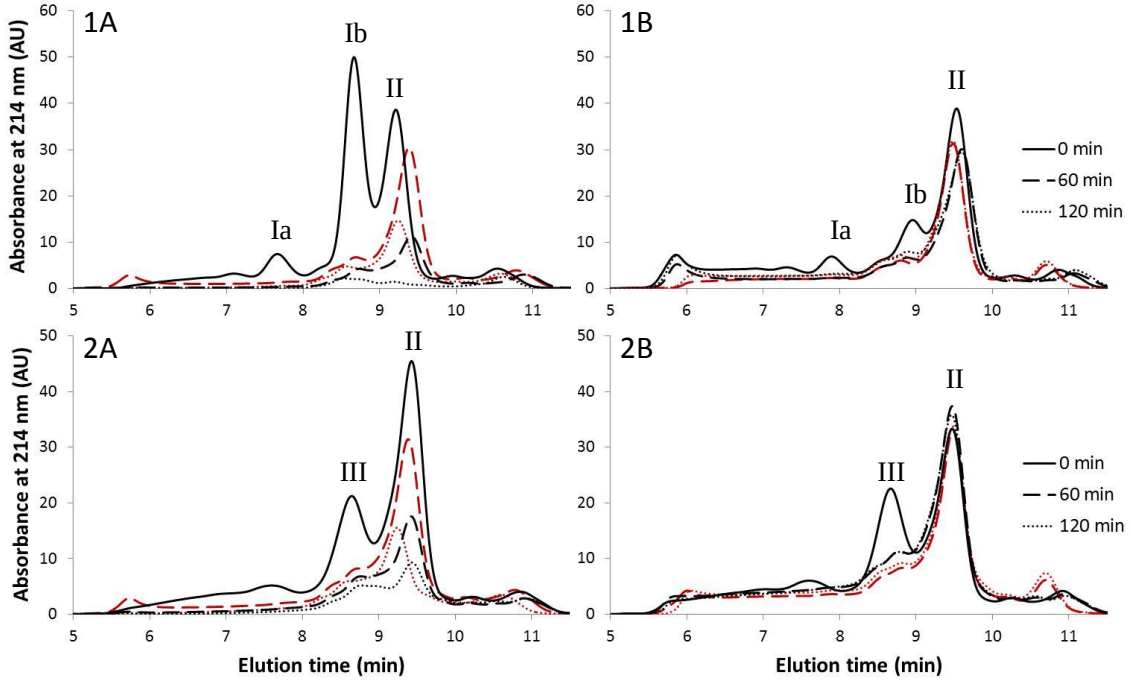
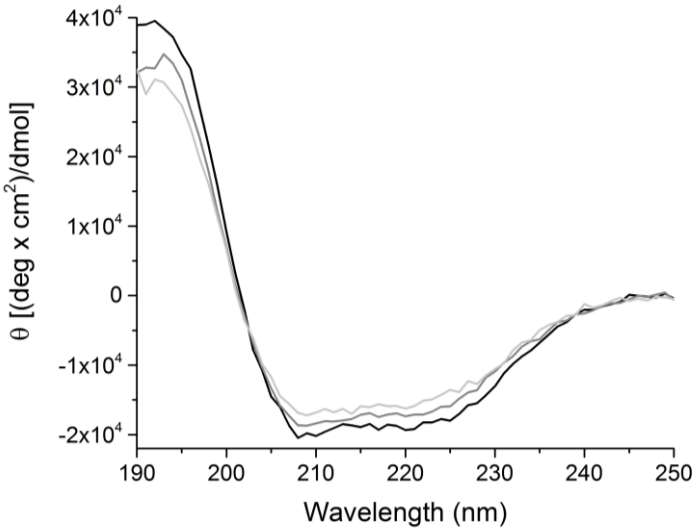


Figure 6. SE-HPLC profile of protein extracts in sodium dodecyl sulfate containing medium (SDS-EP) of mixtures (ratio 2:1) of gliadin and bovine serum albumin (BSA, 1) and gliadin and soy glycinin (2) heated at 100 °C for different times in water (A) and 50% (v/v) ethanol (B). Peaks Ia and Ib are attributed to BSA dimers and monomers respectively. The main peaks of gliadin (II) and soy glycinin (III) are shown. Calculated profiles based on data of the isolated proteins are shown in red. AU, arbitrary units.

Table 1. Proteins extractable in sodium dodecyl sulfate containing medium (SDS-EP, %, with standard deviations between brackets) of bovine serum albumin (BSA), soy glycinin and wheat gluten heated in water at 100 °C for various times with or without pretreatment with 50% (v/v) ethanol (EtPT).

	Heated in water				EtPT and heated in water			
	0 min	6 min	60 min	120 min	0 min	6 min	60 min	120 min
BSA	94 (3)^{a}	5 (0)^{b}	3 (0)^{a}	3 (0)^{b}	84 (9)^{a}	8 (1)^{a}	3 (0)^{a}	4 (0)^{a}
Soy glycinin	97 (4)^{a}	92 (3)^{b}	37 (2)^{a}	33 (1)^{a}	96 (3)^{a}	97 (3)^{a}	34 (2)^{a}	35 (3)^{a}
Wheat gluten	75 (2)^{a}	52 (2)^{a}	31 (1)^{a}	25 (0)^{a}	73 (5)^{a}	45 (4)^{b}	30 (2)^{a}	24 (2)^{a}

Results of the same protein and heating time indicated with the same letter are not significantly different ($P < 0.05$).



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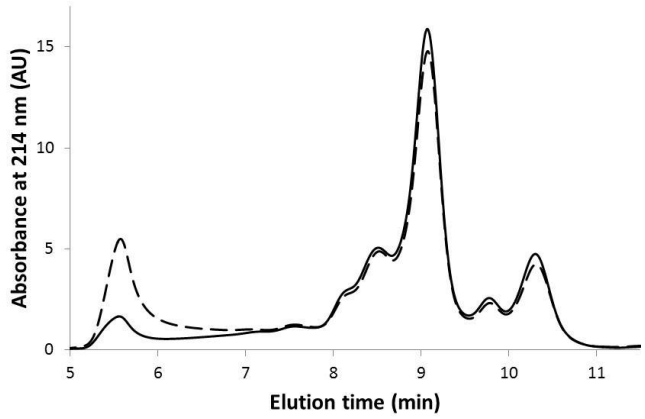
583 **Figure 7.** CD spectra of bovine serum albumin (BSA) in water (–), ethanol-pretreated (EtPT) BSA in
584 water (–) and BSA in 50% (v/v) ethanol (–).

585 **Table 2.** Proteins extractable in sodium dodecyl sulfate containing medium (SDS-EP, in %, with
586 standard deviations between brackets) of a mixture of bovine serum albumin (BSA) with gluten (ratio
587 1:2). Proteins with subscript EtPT have been pretreated with 50% (v/v) ethanol. The mixtures of the
588 proteins have been heated for various times at 100 °C in water.

	SDS-EP (%)			
Sample ratio (1:2)	0 min	6 min	60 min	120 min
BSA/gluten	86 (6)[a]	28 (1)[a]	14 (1)[a]	14 (1)[a]
BSA_{EtPT} /gluten	83 (2)[a]	28 (1)[a]	16 (1)[a]	16 (1)[a]
$BSA/gluten_{EtPT}$	78 (4)[a]	22 (2)[b]	10 (1)[b]	10 (1)[b]

589 Results in the same column indicated with the same letter are not significantly different (P<0.05).



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591 **Figure 8.** SE-HPLC profile of protein extracts in sodium dodecyl sulfate containing medium (SDS-EP) of
592 mixtures (1:2 ratio) of bovine serum albumin (BSA) and gluten (–) or 70% (v/v) ethanol-pretreated
593 gluten (--) heated for 2 min at 100 °C in water. AU, arbitrary units.